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AGE-DEPENDENT DIFFERENCES IN DOPAMINE TRANSPORTER AND VESICULAR MONOAMINE TRANSPORTER-2 FUNCTION AND THEIR IMPLICATIONS FOR METHAMPHETAMINE NEUROTOXICITY

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Abstract

The abuse of methamphetamine (METH) is a serious public health problem because METH can cause persistent dopaminergic deficits in the brains of both animal models and humans. Surprisingly, adolescent post-natal day (PND)40 rats are resistant to these METH-induced deficits while young adult PND90 rats are not. Studies described in this report used rotating disk electrode voltammetry and western blotting techniques to investigate whether there are age-dependent differences in monoamine transporter function in PND38–42 and PND88–92 rats that could contribute to this phenomenon. The initial velocities of dopamine (DA) transport into, METH-induced DA efflux from, and DA transporter (DAT) immunoreactivity in striatal suspensions are greater in PND38–42 rats than in PND88–92 rats. DA transport velocities into vesicles that co-fractionate with synaptosomal membranes after osmotic lysis are also greater in PND38–42 rats. However, there is no difference in vesicular monoamine transporter-2 (VMAT-2) immunoreactivity between the two age groups in this fraction. This suggests that younger rats have a greater capacity to sequester cytoplasmic DA into membrane-associated vesicles due to kinetically upregulated VMAT-2 and also have increased levels of functionally active DAT. In the presence of METH, these may provide additional routes of cellular efflux for DA that is released from vesicles into the cytoplasm and thereby prevent cytoplasmic DA concentrations in younger rats from rising to neurotoxic levels after drug administration. These findings provide novel insight into the age-dependent physiological regulation of neuronal DA sequestration and may advance the treatment of disorders involving abnormal DA disposition including substance abuse and Parkinson's disease.

Keywords

Ageing; Efflux; Vesicle

TEXT

The abuse of methamphetamine (METH) is a serious public health problem with 7.3% of young adults (19–30 years of age) in the United States reporting lifetime illicit use of METH (Johnston et al., 2007b). By way of comparison, the percentages of adolescent 8th, 10th, and 12th graders in the United States reporting lifetime illicit use of METH are 2.7, 3.2, and 4.4%, respectively

(Johnston et al., 2007a). This prevalence of use is concerning because the behavioral consequences of METH differ as a function of age (Vorhees et al., 2005). METH and related stimulants can also cause long-term decreases in markers for monoaminergic neurons (Brown and Yamamoto, 2003). Specifically, multiple high-dose METH administrations cause long-term reductions in striatal dopamine (DA) content, tyrosine hydroxylase activity, and DA transporter (DAT) density in rodents (Hotchkiss et al., 1979; Wagner et al., 1980). These persistent dopaminergic deficits are caused, at least in part, by the METH-induced aberrant accumulation of cytoplasmic DA and the subsequent formation of DA-associated reactive oxygen species (Cubells et al., 1994; Fumagalli et al., 1999); and also appear to occur in human adults who chronically abuse METH (Wilson et al., 1996).

Surprisingly, there appears to be an age-dependent vulnerability to these effects because multiple high-dose METH administrations cause persistent dopaminergic deficits in post-natal day (PND)90 but not PND40 rats (Kokoshka et al., 2000; Truong et al., 2005). PND40 and PND90 rats correspond to human adolescents and young adults, respectively, (Ojeda et al., 1980) and the mechanisms underlying these age-dependent differences are not completely understood. A greater basal striatal DA content in PND90 versus PND40 rats may contribute (Truong et al., 2005). Additionally, the rate of METH-induced DA efflux out of vesicles has been measured in these two age groups and is greater in PND88–92 rats than in PND38–42 rats (Volz et al., 2006a). Both this higher concentration of DA and the ability to release more DA into the cytoplasm may increase the cytoplasmic production of DA-associated reactive oxygen species in PND90 rats compared with PND40 rats after multiple high-dose METH administrations and thus cause persistent dopaminergic deficits. METH also affects a variety of neurotransmitters including serotonin that may also contribute to the effects of the stimulant.

METH-induced alterations in the activity of monoamine transporter proteins have also been reported to contribute to persistent dopaminergic deficits (for review see (Volz et al., 2007b)). The DAT functions to transport DA across the plasmalemmal membrane from the synaptic cleft and into the cytoplasm while the vesicular monoamine-transporter-2 (VMAT-2) sequesters cytoplasmic DA inside neuronal vesicles. Because the DAT and VMAT-2 are the primary regulators of cytoplasmic DA levels, age-dependent differences in transporter function may also underlie the resistance of PND40 rats to METH-induced persistent dopaminergic deficits. Age-dependent differences in the function of the VMAT-2 in cytoplasmic vesicles (i.e., those vesicles that do not remain associated with synaptosomal membranes after osmotic lysis) have been examined (Truong et al., 2005). The present report describes experiments investigating age-dependent differences in the function of VMAT-2 in an entirely different population of vesicles that co-fractionate with synaptosomal membranes after osmotic lysis (referred to herein as membrane-associated vesicles) as well as in the function of the plasmalemmal DAT in adolescent PND38–42 and young adult PND88–92 rats.

Rotating disk electrode voltammetry was used to measure the initial velocities of inwardly directed DA transport into, and outwardly directed METH-induced DA efflux from, rat striatal suspensions as well as the initial velocities of inwardly directed vesicular DA transport into membrane-associated vesicles purified from rat striata as described previously (Volz et al., 2007a; Volz et al., 2006a; Volz et al., 2006b; Volz et al., 2004; Volz and Schenk, 2004). Briefly, untreated and unanesthetized male Sprague-Dawley rats from Charles River Laboratories (Raleigh, NC) were sacrificed by decapitation and striata (but not the nucleus accumbens) were removed. For striatal suspension measurements, each separate measurement was done on a sample that consisted of one rat striatum that was chopped with a razor blade, placed in 500 μ l of DAT assay buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 16 mM sodium phosphate, 1.4 mM MgSO₄, 11 mM dextrose, pH 7.4, and maintained at 37 °C), and disrupted by repetitive pipetting. A rotating disk electrode was lowered into the resulting striatal suspension and rotated at 2000 rpm. A +450 mV potential was applied and a detection current baseline was

established. For experiments measuring DA transport, an aqueous DA solution was then added to the striatal suspension resulting in a final DA concentration of 1.0 μM . Alternatively, for experiments measuring METH-induced DA efflux, an aqueous ($\pm$)-METH solution was added *in vitro* to the striatal suspension resulting in a final METH concentration of 40 μM .

For membrane-associated vesicle measurements, each separate measurement was conducted using a sample that consisted of both striata from a rat that were homogenized and then subjected to an osmotic lysis and differential centrifugation protocol to isolate a membrane-associated vesicle pellet as described previously (Volz et al., 2007a). The membrane-associated vesicle pellet was resuspended in 500 μl of VMAT-2 assay buffer (25 mM HEPES, 100 mM potassium tartrate, 0.05 mM EGTA, 0.1 mM EDTA, 2 mM Mg^{2+} -ATP, pH 7.5, and maintained at 37 $^{\circ}\text{C}$) and a detection current baseline was established as above. An aqueous DA solution was then added resulting in a final DA concentration of 1.5 μM . The resulting continuous real-time current outputs from all of the above rotating disk electrode measurements were recorded and the initial velocities of inwardly directed DA transport and outwardly directed METH-induced DA efflux were calculated from the linear slope of the initial apparent zero-order portion of a plot of DA concentration versus time as described previously (Earles et al., 1998; McElvain and Schenk, 1992; Volz et al., 2007a; Volz et al., 2006b). Following rotating disk electrode voltammetry, SDS-polyacrylamide gel electrophoresis and western blot analysis were performed on the membrane-associated vesicle fractions and striatal suspensions as previously described (Riddle et al., 2002; Volz et al., 2007a).

The results from these experiments revealed the heretofore unreported findings that the initial velocities of both inwardly directed DA transport into, and outwardly directed METH-induced DA efflux from, striatal suspensions are greater in adolescent PND38–42 rats than in young adult PND88–92 rats (Figure 1A). These data suggest the possibility that the striatal suspensions of PND38–42 rats contain more functionally active DAT than suspensions prepared from the striata of PND88–92 rats. This idea is supported by western blot analysis, as DAT immunoreactivity in striatal suspensions is greater in the adolescent age group (Figure 1B).

There are disparities within the literature concerning age-dependent differences in both DAT levels and function in rat striatum. One study reported a *decrease* in the B_{max} of the binding of the DAT ligand, [^3H]GBR 12935, in PND30 versus PND90 rat striatal synaptosomes (Gordon et al., 1995) while a later study found an *increase* in the B_{max} of the binding of [^3H]GBR 12935 in PND50 versus PND90 rat striatal synaptosomes (Moll et al., 2000). The B_{max} of the binding of the DAT ligand, [^3H]BTCF, also decreases with age (Patel et al., 1994). In contrast, another study concluded that there is no difference in the binding of the DAT ligand, [^3H]WIN 35428, or in [^3H]DA transport in PND40 and PND90 rat striatal synaptosomes (Kokoshka et al., 2000). There is also no difference in binding of the DAT ligand, [^{125}I]RTI 121, in PND37 and PND67 rat striatal slices (Collins et al., 2004).

Differences in the experimental paradigms and techniques used to assess striatal DAT levels and function may contribute to these apparently conflicting results. For example, DAT immunoreactivity (Figure 1B) and function (Figure 1A) in striatal *suspensions* (which are essentially a homogenized and unpurified whole striatal synaptosomal preparation) are greater in PND38–42 rats than in PND88–92 rats. However, there is no difference between these two age groups when DAT immunoreactivity and function are assessed in purified striatal *synaptosomes* (data not shown) which confirms an earlier report (Kokoshka et al., 2000). One explanation for these differences may be a preferential loss of vulnerable (active) DAT during the preparation of purified striatal *synaptosomes* from young rats. Alternatively, the differences in DAT levels and function seen in striatal *suspensions* may be distributed among the various

subcellular fractions during preparation and purification of *synaptosomes* such that they are no longer detectable.

The finding of Moll et al. (2000) of an increase in the $B_{\max}$ of the binding of [^3H]GBR 12935 in PND50 versus PND90 rat striatal synaptosomes is in agreement with the DA transport (Figure 1A), METH-induced DA efflux (Figure 1A), and DAT immunoreactivity (Figure 1B) results reported in the present study. Taken together, the findings from Moll et al. (2000) and the present report suggest that adolescent PND38–42 rats possess an additional population of functionally active DAT in the striatum that is not present in young adult PND88–92 rats. Age-dependent differences in brain DAT levels have also been reported in humans with the putamen of human adolescents (mean age 9 ± 2 years) having higher levels of DAT immunoreactivity than the putamen of human young adults (mean age 30 ± 3 years) (Haycock et al., 2003).

The increased levels of functionally active DAT in the striata of PND38–42 rats compared with PND88–92 rats have important implications with regard to METH-induced persistent dopaminergic deficits. Outwardly directed METH-induced DA efflux from striatal neurons is mediated by the DAT (for review see (Fleckenstein et al., 2007)) and the increased DAT levels in PND38–42 rats may provide additional routes of cellular efflux for DA that is released from vesicles into the cytoplasm by METH. Supporting this idea, DA efflux out of striatal slices caused by the METH analog, amphetamine, is also greater in PND30 rats than in PND90 rats (Gordon et al., 1995). These results suggest that the increased METH-induced striatal DA efflux in PND38–42 rats, facilitated by increased levels of functionally active DAT, may act to prevent cytoplasmic DA concentrations in younger rats from rising to neurotoxic levels after multiple high-dose METH administrations and may help to explain why adolescent PND40 rats are resistant to METH-induced persistent dopaminergic deficits.

Age-dependent differences in the function of the VMAT-2 in cytoplasmic vesicles have also been reported and indicate that PND90 rats have more VMAT-2-containing cytoplasmic vesicles than do PND40 rats (Truong et al., 2005). However, PND90 rats also have greater total tissue DA content (Truong et al., 2005) and more METH-induced DA efflux out of vesicles and into the cytoplasm (Volz et al., 2006a) than PND40 rats. Moreover, multiple high-dose METH administrations cause both greater acute and persistent decreases in the function of the VMAT-2 in cytoplasmic vesicles in PND90 rats than in PND40 rats (Truong et al., 2005). Thus, METH increases cytoplasmic DA concentrations through vesicular DA efflux to a greater extent in PND90 rats while concurrently causing greater impairment in the DA sequestration ability of cytoplasmic vesicles in PND90 rats. This is likely one of the reasons that PND90 rats, but not PND40 rats, are susceptible to the persistent deficits caused by multiple high-dose METH administrations.

The present report extends these findings by describing experiments that were conducted to investigate age-dependent differences in the function of the VMAT-2 in an entirely different population of vesicles, the membrane-associated vesicles. Unlike cytoplasmic vesicles, these vesicles remain associated with synaptosomal membranes after osmotic lysis, have a sigmoidal response curve, and have DA transport kinetics that display a unique positive cooperativity that is not seen in any other vesicular neurotransmitter transporter. They also have the ability to sequester large amounts of DA under conditions of elevated cytoplasmic DA concentration as may occur after treatment with METH (Volz et al., 2007a). As such, membrane-associated vesicles may function as a “DA sink” to prevent cytoplasmic DA concentrations from rising to aberrant levels (Volz et al., 2007a).

As seen above with DAT-mediated transport, the initial velocities of inwardly directed VMAT-2-mediated DA transport into membrane-associated vesicles are greater in adolescent PND38–42 rats than in young adult PND88–92 rats (Figure 2A). However, there is no

difference in VMAT-2 immunoreactivity in membrane-associated vesicles isolated from PND38–42 and PND88–92 rats (Figure 2B). This finding confirms an earlier study that also found no difference in the VMAT-2 immunoreactivity of the membrane-associated vesicle subcellular fractions of PND40 and PND90 rats (Truong et al., 2005).

An increase in vesicular DA transport velocities (Figure 2A) with no change in VMAT-2 density (Figure 2B) suggests that the VMAT-2 is kinetically upregulated in the membrane-associated vesicles of PND38–42 rats compared with PND88–92 rats (i.e., the vesicles of PND38–42 rats sequester a larger quantity of DA due to an increase in the rate at which the VMAT-2 transports DA). This is the first report of age-dependent VMAT-2 kinetic regulation. The kinetic upregulation of the VMAT-2 in the membrane-associated vesicles of PND38–42 rats may act to prevent cytoplasmic DA concentrations from rising to neurotoxic levels after multiple high-dose METH administrations and may thus be another factor underlying the resistance of adolescent PND40 rats to METH-induced persistent dopaminergic deficits. The VMAT-2 in membrane-associated vesicles can also be kinetically upregulated by *in vivo* pharmacological treatment with methylphenidate (Volz et al., 2007a) which has also been shown to prevent METH-induced persistent dopaminergic deficits (Sandoval et al., 2003).

Taken together, the age-dependent differences in monoamine transporter function suggest that adolescent rats are resistant to the persistent dopaminergic deficits caused by METH because: 1) younger rats have greater capacity to sequester cytoplasmic DA into membrane-associated vesicles due to kinetically upregulated VMAT-2, 2) younger rats have increased levels of functionally active DAT which provide additional routes of cellular efflux for DA that is released from vesicles into the cytoplasm by METH, and 3) younger rats have fewer VMAT-2-containing cytoplasmic vesicles which results in less METH-induced DA efflux from vesicles into the cytoplasm. All of these factors may act to prevent cytoplasmic DA concentrations from rising to neurotoxic levels after multiple high-dose METH administrations in younger rats. Abnormal cytoplasmic DA accumulation also likely contributes to the development of Parkinson's disease (Jenner, 1998) and the results presented here may provide insight into the cause and treatment of this disorder as well.

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Figure 1.

The initial velocities of inwardly directed DA transport into, and outwardly directed METH-induced DA efflux from, striatal suspensions (Panel A) as well as striatal suspension DAT immunoreactivity (Panel B) are greater in adolescent PND38–42 rats than in young adult PND88–92 rats. Each column represents the mean $\pm$ SEM of four separate measurements (i.e., each measurement was done on tissue prepared from a different rat). An asterisk indicates a statistical difference, $p < 0.05$ via a two-tailed paired t -test, between DA transport velocities ($t = 3.65$, $df = 3$), DA efflux velocities ($t = 7.17$, $df = 3$), or DAT immunoreactivity ($t = 6.21$, $df = 3$) in PND38–42 and PND88–92 rats. Molecular weight (in kilodaltons) is shown to the left of the representative blot.

Figure 2.

The initial velocities of inwardly directed DA transport into membrane-associated vesicles (Panel A), but not VMAT-2 immunoreactivity in membrane associated vesicles (Panel B), are greater in adolescent PND38–42 rats than in young adult PND88–92 rats. Each column represents the mean $\pm$ SEM of four separate measurements (i.e., each measurement was done on tissue prepared from a different rat). An asterisk indicates a statistical difference, $p < 0.05$ via a two-tailed paired t -test, between DA transport velocities ($t = 14.7$, $df = 3$) in PND38–42 and PND88–92 rats. Molecular weight (in kilodaltons) is shown to the left of the representative blot.